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Research Note

Supercritical Fluid Extraction of Aflatoxin M_1 from Beef Liver†

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ABSTRACT

Supercritical fluid extraction (SFE) and a pressurized-fluid-extraction process were applied for the removal of aflatoxin M_1 from beef liver samples. Various pressures, temperatures, quantity of supercritical carbon dioxide, and organic modifiers were investigated to optimize the extraction methods. Organic modifier was found to be essential for quantitative recovery of aflatoxin M_1. Extracts were cleaned up by solid-phase extraction and were analyzed via high-performance liquid chromatography coupled with fluorescence detection of the trifluoroacetic acid derivative. Solvent-modified carbon dioxide SFE achieved recoveries comparable to an AOAC-approved method involving organic solvent extraction. SFE allowed the traditional amounts of sample and organic solvent to be reduced. Also, the supercritical-fluid extraction permitted the use of carbon dioxide modified with acetonitrile: methanol (2:1) to replace methylene chloride as the organic solvent for the extraction step.

Key words: Supercritical-fluid extraction, aflatoxin M_1, beef, liver, organic modifier

Aflatoxin-contaminated agricultural commodities (corn, peanuts, and cottonseed) occur worldwide. Aflatoxins are produced primarily by *Aspergillus flavus* or *Aspergillus parasiticus.* They are extremely potent carcinogens and efforts are necessary to keep them from entering our food supplies. One option is for farmers to divert a contaminated crop from consumer markets and utilize it for animal feed. However, this may result in animal loss due to mycotoxins in the food, reduced resistance of the animals to other diseases, and the possibility of producing contaminated edible meats, milk, and dairy products *(17).*

Several reviews *(4, 13, 15)* have summarized the aflatoxin B_1 and M_1 concentrations detected in edible products such as milk, eggs, pork, chicken, and turkey. Also, Coulter et al. *(2)* reported that aflatoxins have been found in liver biopsies of children of women in countries that annually have aflatoxin-contaminated grain. The major portion of the ingested aflatoxin is excreted in animal wastes within 24 h; however, small quantities of the toxin are retained in the tissues. Of these, the liver is the target organ of aflatoxicosis, and it has been the subject of numerous investigations *(18).*

Recently, supercritical-fluid extraction (SFE) has been shown to be an alternative to conventional organic-solvent extraction for aflatoxins B_1, B_2, G_1, and G_2. However, this prior research focused on matrices such as peanut meal, corn, and grain dust *(5, 8, 14, 20, 21).* In this study, SFE was evaluated as an extraction technique for trace levels of incurred aflatoxin M_1, which is the major metabolite found in beef liver *(18).* The approved official organic-solvent-based extraction requires 100-g liver samples and uses 200 ml of methylene chloride. The extract is then cleaned up via silica column chromatography employing approximately 100 ml of methylene chloride along with other organic solvents *(1).* In this study, various sample sizes of liver tissue were extracted to see how small a sample could be used in the extraction while maintaining analysis reproducibility and precision. It was deduced that 20-g samples were suitable for this study. The aim of this research was then to determine the parameters that would allow the SFE of aflatoxin M_1 from 20-g liver samples and to compare the recoveries obtained by SFE with those produced by organic-solvent extraction.

MATERIALS AND METHODS

Liver samples

One-sixth of a whole bovine liver was ground and blended in a Robot Coupe food processor (Robot Coupe, USA, Inc., Ridgeland, MS) for 1 min. This material was then passed through a no. 20 sieve, reblended for an additional minute and divided into 100-g subsamples. These were stored at −16°C until used.

Solvent extraction

Liver (20 g) and 4 ml of a 20% citric acid solution were added to a 250-ml glass-stoppered Erlenmeyer flask, mixed, and allowed to react for 5 min. Then celite (4 g) and methylene chloride (60 ml) were added and mixed thoroughly. The flask was shaken on a

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† Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

wrist-action shaker for 30 min. The mixture was filtered through Schleicher & Schuell fast-flow filter paper (Schleicher & Schuell, Inc., Keene, NH) into a 250-ml flask containing Na_2SO_4 (5 g). This was shaken by hand, intermittently, for 2 min and then filtered through a glass-fiber filter. The extract was cleaned up on a 6-ml silica Mega Bond Elut column (Varian, Harbor City, CA) which had been conditioned by passing hexane (5 ml) through it under vacuum. An aliquot (10 ml) of the extract was diluted with hexane (10 ml) before use of solid-phase extraction (SPE) for sample cleanup. The extract was applied to the SPE column; it was then eluted consecutively with hexane (5 ml), diethyl ether (5 ml), ethyl acetate (5 ml), and acetonitrile (15 ml). Aflatoxin M_1 eluted in acetonitrile and was concentrated to dryness at 60°C under an N_2 stream. The dried sample was then derivatized with trifluoroacetic acid to convert aflatoxin M_1 to M_{2a} for enhanced detection via fluorescence in the reversed-phase high-performance liquid chromatography (HPLC) eluent *(11)*. The derivatized sample was dissolved in 1 ml of the HPLC mobile phase, and 100 µl injected for analysis. Quantitation was performed via high-performance liquid chromatography using a previously described *(20)* method. The mobile phase in this case was water/acetonitrile/isopropyl alcohol (88/6/6 vol%) at a flow rate of 1 ml/min.

Supercritical fluid extraction

Prior to extraction, 4 ml of a 20% citric acid solution was pipetted into a 150-ml beaker containing 20 g of liver. This mixture was stirred, allowed to stand for 10 min, mixed with 15 g of an extraction-enhancing agent *(9)*, Chem-Tube Hydromatrix (Analytichem International, Harbor City, CA), and then placed in the extraction cell. The basic SFE device used in this study was similar to a previously described unit in construction and operating principle *(6)*. However, as shown in Figure 1, it was modified by plumbing an HPLC pump (Model SD-1, Rainin Instrument Company Inc., Woburn, MA) into the system so that a modifier could be added to the CO_2 for extraction enhancement. SFE was conducted in a vertically positioned extraction cell that was constructed from 316 stainless-steel tubing, 51 by 1.7 cm i.d. Various pressures (5,000 to 10,000 psig), temperatures (80 to 150°C), quantity of CO_2 (100 to 900 liters delivered at 5 liters/min, NSTP) and organic modifiers ($CHCl_3$, ACN:MeOH) were investigated for aflatoxin M_1 extraction. The extracts were collected in 250-ml round-bottom flasks and concentrated to an oily residue below 40°C at reduced pressure prior to SPE.

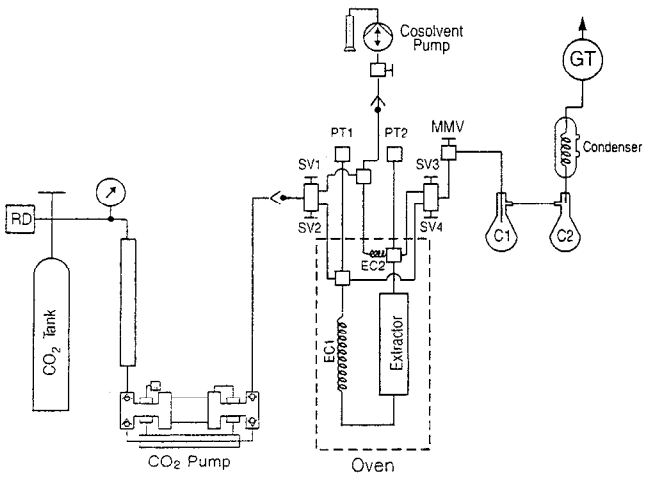

FIGURE 1. *Schematic of the supercritical fluid extraction device: RD, relief disk; SV, switching valve; PT, pressure transducer; EC, equilibration coil; MMV, micrometering valve; C, collection flask; GT, gas totalizer.*

The supercritical-fluid extracts were dissolved in $CHCl_3$ (100 ml) and filtered through Na_2SO_4 to remove any water that might be present. An aliquot (10 ml) was then subjected to SPE as described above for sample cleanup prior to HPLC analysis.

RESULTS AND DISCUSSION

Earlier reports on supercritical-fluid extraction of aflatoxins from agriculturally derived substrates show the necessity of solvent-modified carbon dioxide for quantitative recovery *(5, 8, 14, 20, 21)*. In the present study, we are reporting the first SFE of an incurred aflatoxin M_1 from a biological tissue, beef liver. This work is an extension of the SFE methodology reported by our group involving ground corn as the matrix *(20)*. In contrast to infested grain samples, aflatoxin contamination is not localized, but it is distributed evenly throughout the tissue matrix *(16, 18)*.

Initial extractions were performed with neat CO_2 at 5,000 and 10,000 psig at 80°C. Dynamic SFE at the higher pressure yielded 0.1 ppb of aflatoxin M_1, which was about one-third the recovery obtained via organic-solvent extraction. Because of this low recovery, an SFE with a cosolvent was attempted. The extraction pressure in this case was lowered to 5,000 psig and a modifier consisting of a 2:1 mixture of ACN:MeOH (previously used on ground corn) was utilized to enhance the recovery of the aflatoxin. There is good reason to use this particular binary modifier. The structures of aflatoxin B_1 and its metabolite, aflatoxin M_1, can be characterized by solubility parameters of 12.7 and 14.3 $cal^{1/2}/cc^{3/2}$, respectively, as calculated by the group-contribution methods of Fedors *(7)*. These solubility parameters approximate those exhibited by acetonitrile and methanol, 12.1 and 14.5 $cal^{1/2}/cc^{3/2}$ respectively, the components of the above-described binary modifier. Therefore, it is not surprising that this particular modifier combination enhances the extraction of aflatoxins. During the course of the extraction, 250 ml of this modifier was added to the CO_2 stream, producing an extract that was too complex to be cleaned up by SPE.

Due to this result, we attempted to extract the mycotoxin from the liver by initially adding some organic solvent to the extraction cell and statically holding it before initiating a purge with supercritical CO_2. Two different modifiers were investigated: (A) ACN:MeOH, 2:1, and (B) $CHCl_3$. The HPLC pump (Fig. 1) was used to deliver modifier (80 ml) into the extraction cell. The cell was heated to 150°C and then pressurized with CO_2 to 5,000 psig. A static hold (30 min) was then enacted, followed by dynamic SFE with neat CO_2 (100 liters at NSTP as measured on a dry test meter) to purge the organic solvent from the extraction cell into the receiver. These extracts also proved too complex and were not amenable to SPE cleanup.

Dynamic SFE at a higher pressure (8,500 psig) and lower modifier (ACN:MEOH, 2:1) concentration (65 ml) was then attempted, resulting in quantitative recovery of aflatoxin M_1 from the liver. The amount of modifier used in this case for the extraction was only 3.3 vol%. Table 1 compares the recoveries of aflatoxin M_1 using both the above-described SFE process and organic-solvent extraction. The average recovery obtained by supercritical-fluid extraction using a cosolvent compares favorably to that via organic-

TABLE I. *Recoveries of aflatoxin M_1: solvent extraction versus supercritical fluid extraction*

Trial no.	Recovery aflatoxin M_1 (ppb) by: Organic solvent[a]	Supercritical fluid extraction[b]
1	0.30	0.32
2	0.30	0.29
3	0.32	0.40
4	0.31	0.40
5	—[c]	0.36
6	—	0.37
Average	0.31	0.36
(% relative standard deviation)	(3.11)	(12.37)

[a] CH_2Cl_2.
[b] 8500 psig, 80°C, 900 liters of CO_2, 65 ml of ACN:MeOH (2:1).
[c] Not determined.

solvent extraction, but the resultant relative standard deviation (RSD) is about 4× larger in the SFE case. However, this RSD (12%) is well below the typical value range (20 to 32%) that has been determined acceptable for aflatoxins at the ppb level, as shown by collaborative studies conducted by the AOAC *(10)*. We feel this can probably be improved upon by adjusting the extraction parameters to yield an even cleaner extract with less extraneous interfering coextractives.

It should be noted that Wu et al. *(21)* reported extracting aflatoxins (B_1, B_2, G_1, and G_2) from peanut meal with a highly pressurized fluid-extraction process. They stated that true supercritical conditions were not maintained because of the proportion of modifier in the extraction medium, but they noted that the pressurized CO_2-methanol system was much better than neat supercritical CO_2 for aflatoxin extraction. This technique then is very similar to static SFE with modifier added to the extraction cell prior to pressurization with CO_2. However, the main difference between these two techniques is in the relative amount of modifier added to the extraction cell. The method of Wu et al. *(21)* is also similar to the technique used by Cui and Olesik *(3)* and previous work of the authors *(19)*, so-called enhanced-fluidity techniques, in which a large proportion of modifier is mixed with CO_2 for use as an extraction medium. However, the presently reported study is different from the recently developed method of accelerated solvent extraction *(12)* in that the fluid (gas) is an active agent in affecting the extraction of the target analyte, rather than simply a pressure-transmitting medium.

This is the first report of a supercritical fluid extraction of incurred aflatoxin M_1 at low levels from beef liver. Quantitative recovery of sub-ppb levels of aflatoxin M_1 can be achieved via this technique. The described method however offers a viable alternative to analytical methods employing large quantities of organic solvents. The SFE technique, as applied to aflatoxin analysis, can be performed on smaller samples, which also reduces organic solvent consumption when compared to the AOAC-approved method. In this particular case, SFE can be used to eliminate methylene chloride from the extraction step. Possible future studies to which this technique would be applicable are the determination of aflatoxin M_1 in other edible tissues, such as heart, spleen, muscle, and kidney.

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